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AUTHOR(S):

Sutani, Akitoshi; Ueno, Masahiro; Nakagawa, Satoshi; Sawayama, Shigeki

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Melon aroma-producing yeast isolated from coastal marine sediment in Maizuru Bay, Japan

Akitoshi Sutani[1] · Masahiro Ueno[2] · Satoshi Nakagawa[1]· Shigeki Sawayama[1]

(Mail) Shigeki Sawayama
sawayama@kais.kyoto-u.ac.jp

[1] Laboratory of Marine Environmental Microbiology, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

[2] Maizuru Fisheries Research Station, Field Science Education and Research Center, Kyoto University, Kyoto 625-0086, Japan

Abstract Researches on marine fungi and fungi isolated from marine environments are not active compared with those on terrestrial fungi. The aim of this study was isolation of novel and industrially applicable fungi derived from marine environments. In this study, 16 fungus-like strains, MS1–MS16, were isolated from coastal marine sediment in Maizuru Bay, Japan, under aerobic culture conditions. Phylogenetic analysis of 18S rRNA gene sequences indicated that 10 isolates belonged to Ascomycota, five isolates belonged to Sordariomycetes, two were Dothideomycetes, and three were Saccharomycetes. Liquid and agar potato dextrose cultures of strains MS1 and MS2 isolated from the coastal sediment released a melon-like aroma. Gas chromatography analysis suggested that strains MS1 and MS2 produce four major chemicals associated with a melon aroma, cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal. The sequence analyses of the 26S rRNA domains 1/2 (D1/D2) and internal transcribed spacer (ITS) regions indicated that strains MS1 and MS2 were phylogenetically identified as *Geotrichum candidum*, a well-known yeast used as a cheese starter. These results suggest the future isolations of novel and functional fungi from marine environments.

Key words Marine fungi· Melon-aroma· Yeast· Coastal sediment· Phylogenetic analysis

Introduction

Fungi include a variety of industrially useful species such as *Hypocrea jecorina* (*Trichoderma reesei*) and *Saccharomyces cerevisiae*. *H. jecorina* produces and secretes a large amount of cellulase, and is a useful industrial enzyme producer. *S. cerevisiae* has long been used as an ethanol producer [1, 2]. Terpene glycosides in grapes were reported to be hydrolyzed to free volatile terpene aroma compounds by yeasts during the aging of wines [3]. Ester levels in Bordeaux red wines were strongly influenced by yeast strains [4]. *Kluyveromyces lactis* and *S. cerevisiae* were two potent deacidifying and volatile sulphur-aroma producing yeasts of the cheese ecosystem [5]. *S. cerevisiae* were reported to produce the aroma chemicals of 3-(methylthio)-1-propanol and 3-(methylthio)-propylacetate using L-methionine as sole nitrogen source [6]. Yeasts have a close relationship with flavors and aromas of wines and cheeses.

Marine organisms are thought to be excellent bioresources owing to their production of many useful compounds [7]. Approximately 150–200 new compounds are isolated annually from marine fungi [8]. Marine fungi that produce antimicrobial metabolites have been screened [9]. New prenylxanthones were detected from the deep-sea sediment-derived fungus *Emericella* sp., which was isolated from the sediment (3,258 m) of the South China Sea [10]. New polyketides were detected from the deep-sea sediment-derived fungus *Aspergillus* sp., which was isolated from the hydrothermal vent (2,255 m, temperature 114 ºC) in the

southwest Pacific [11]. There may be many uncultured fungal strains that produce useful compounds in marine environments.

Isolated marine fungi have been classified into the phyla Chytridiomycota, Oomycota, Basidiomycota, and Zygomycota. A marine chytridial parasitoid of dinoflagellates has been identified as a new genus and species, *Dinomyces arenysensis* [12]. A total of 31 fungi isolates were recovered from seawater and sediment samples from the Pearl River Delta (China), and most belonged to the phyla Ascomycota and Basidiomycota [13]. Ninety-eight fungal strains were isolated from two samples of the marine sponge *Dragmacidon reticulatum* using six different culture media, and 64 distinct fungal ribotypes that belonged to 24 genera of Ascomycota and Zygomycota were obtained [14]. An analysis of internal transcribed spacer (ITS) sequences revealed that 101 phenotypically different fungal isolates obtained from 11 sponge samples collected in King George Island, Antarctica belong to the phylum Ascomycota [15]. There may be many uncultured fungal strains representing a wide range of taxa in marine environments.

The definition of marine fungi is problematic. Marine Ascomycetes have a wide salinity tolerance, including low-salinity conditions, and are less conditioned by the available substrate [16]. The effects of seawater concentration on hyphal growth and antimicrobial metabolite production in marine fungi have been studied using 0%, 50%, and 100% seawater-based culture medium [9]. The aim of this study was isolation of novel and

77 industrially applicable fungi derived from marine environments. In this study, fungi were
78 isolated from coastal marine sediment in Maizuru Bay, Japan. Fungi derived from marine
79 sediment habitats were isolated with 100% seawater-based culture medium. Phylogenetic
80 analyses of the 18S rRNA gene, 26S rRNA domains 1 and 2 (26S rRNA D1/D2), and ITS 1/2
81 regions were conducted to identify the isolated fungal strains.
82
83 **Materials and methods**
84
85 **Sampling and isolation**
86
87 A coastal marine surface sediment sample (0–5 cm in depth) was collected using a
88 Smith-McIntyre sediment sampler from a site in Maizuru Bay (35°29'41.1"N 135°22'05.2"E;
89 July 19, 2013) in Kyoto prefecture, Japan. The sediment sample was spread on potato
90 dextrose (PD) agar plates based on seawater, and incubated at 25 °C. Colonies that had a
91 fungus-like appearance were selected for further isolation procedures. The isolation procedure
92 was conducted several times to obtain a pure colony appearance using PDA agar plates.
93 Isolated fungus-like strains were designated strains MS1–MS16.
94
95 **Phylogenetic analyses**
96

Sequence analyses of the 18S rRNA gene, 26S rRNA D1/D2, and ITS regions were conducted. Isolated fungal strains were incubated in PD liquid medium at 30 °C and 120 rpm of shaking for 48 h, and cells were collected by centrifugation (15,000 × *g* for 10 min). Total DNA was extracted from fungus cells using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). DNA fragments of the 18S rRNA gene, 26S rRNA D1/D2, and ITS regions were amplified by polymerase chain reaction with SapphireAmp® Fast PCR Master Mix (Takara Bio, Otsu, Japan).

PCR amplification conditions for the 18S rRNA gene were 40 cycles each of 98 °C for 5 s, 50 °C for 5 s, and 72 °C for 15 s using the specific primer set NS1 and Fungi18S-R (Table 1) [17]. PCR amplification conditions for the 26S rRNA D1/D2 region were 40 cycles each of 98 °C for 5 s, 56 °C for 5 s, and 72 °C for 15 s using the specific primer set NL1and NL4 (Table 1) [18]. PCR amplification conditions for the ITS regions were 40 cycles each of 98 °C for 5 s, 53 °C for 5 s, and 72 °C for 15 s using the specific primer set ITS1 and ITS4 (Table 1) [19].

PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany), and were sequenced using the Applied Biosystems 3730 xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Database searches for similar sequences were conducted using the BLASTN application available at the NCBI website. A multiple sequence alignment was performed using the Clustal Omega program

available at the EMBL-EBI website. A phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA6.0 [20] with 1,000 bootstrap replicates. *Smittium imitatum* or *Dipodascus tetrasporeus* was used as an outgroup. The sequences of the 18S rRNA gene reported in this study have been deposited in the DDBJ/EMBL/GenBank databases under the accession numbers LC032042 (strain MS1) to LC032051 (strain MS16), 26S rRNA D1/D2 region sequences under LC032052 (strain MS1) to LC032053 (strain MS2), and ITS region sequences under LC032054 (strain MS1) to LC032055 (strain MS2).

Gas chromatography analysis

Isolated strains MS1 and MS2 were incubated in the PD liquid medium based on seawater at 30 °C and 120 rpm of shaking for 7 d in 50-ml vials. Head space gases in the vials were injected into a gas chromatography (GC) apparatus of GC-2010 (Shimadzu, Kyoto, Japan). The column was a ZB-WAX (0.53 mm, 30 m, and 1.0 μm). The oven temperature was controlled from 60 °C to 200 °C at a rate of 1 °C min^{-1}. Helium was supplied as a mobile phase at 10 ml min^{-1}. GC analyses of standard chemicals associated with a melon aroma were conducted, including cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal.

Results

Biodiversity of fungous strains isolated from the marine sediment collected at Maizuru Bay

Fig. 1

Sixteen fungus-like strains were isolated from the Maizuru Bay marine sediment sample using PD agar plates under aerobic conditions at 25 °C (Fig. 1). Phylogenetic analysis using 18S rRNA gene sequences indicated that 10 isolates (MS1–MS3, MS6, MS8–MS12, and MS16) belong to Ascomycota (Fig. 2). Strains MS1–MS3 belong to Saccharomycetes, strains MS10 and MS16 belong to Dothideomycetes, and strains MS6, MS8, MS9, MS11, and MS12 belong to Sordariomycetes. Strains MS1–MS3 were related to species in the genus *Geotrichum*, strains MS10 and MS16 were related to species in the genera *Fenestella* and *Pyrenochaeta*, and strains MS6, MS8, MS9, MS11, and 12 were related to species in the genus *Hypocrea*. Sequence similarities for the 18S rRNA gene were 95.9% and 98.5% between strain MS1 and *G. candidum* (X69842) and between strain MS2 and *G. candidum* (X69842), respectively.

Fig. 2

Identifications of the volatile substances corresponding to culture aroma

Fig. 3

Strains MS1 and MS2 produced a melon-like aroma on agar PD plates and in liquid PD

155 culture medium. The cells of strains MS1 and MS2, related to the genus *Geotrichum*, had a
156 yeast-like form (Fig. 3). The cell length of strain MS1 was 7–9 μm, and that of strain MS2
157 was 7–13 μm. Gas chromatograms of strains MS1 and MS2 are shown in Fig. 4. GC analysis
158 indicated that strains MS1 and MS2 produce cis-3-hexen-1-ol, cis-6-nonenal,
159 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal in the liquid PD culture. GC chromatograms
160 show that there are additional volatile compounds in the head space gases of strains MS1 and
161 MS2. The chromatograms of strains MS1 and MS2 indicate a similar pattern. Fig. 4
162

163 **Fingerprinting of the yeast strains producing the melon aroma**

164

165 Phylogenetic analysis of strains MS1 and MS2 based on the 26S rRNA D1/D2 region
166 sequence indicated that these two strains form an monophyletic clade with *G. candidum*
167 CBS178.71, *G. candidum* CBS607.85, *G. candidum* CBS11628, and *G. candidum* CBS11616
168 (Fig. 5). Sequence similarities for the 26S rRNA D1/D2 region were 99.3% and 99.6%
169 between MS1 and *Galactomyces candidum* CBS11616 (JN974264) and between MS2 and *G.*
170 *candidum* CBS11616 (JN974264), respectively. *Geotrichum phurueaensis*, *Galactomyces*
171 *pseudocandidum*, *Geotrichum europaeum*, and *Galactomyces geotrichum* form an
172 monophyletic clade. *Galactomyces reessii* and *Galactomyces citri-aurantii* form an
173 monophyletic clade. The sequence analyses of strains MS1 and MS2 based on the 26S rRNA

D1/D2 region showed that these two strains were phylogenetically identified as *G. candidum* (Fig. 5).

Fig. 5

Sequence analysis of the ITS region showed that strain MS1 forms a clade with *G. candidum* Tom1 and *G. candidum* 282A (Fig. 6). Sequence similarities for the ITS region were 99.6% and 99.6% between strain MS1 and *G. candidum* Tom1 (KF298071) and between strain MS1 and *G. candidum* 282A (KF669518), respectively. The sequence of strain MS1 was relatively similar to those of *G. candidum* L19PB, *G. candidum* L13PC, and *G. candidum* L20B. Strain MS2 formed a clade with *G. candidum* Thu1. The sequence similarity for the ITS region was 98.6% between strain MS2 and *G. candidum* Thu1 (KF298070). The sequence of strain MS2 was relatively similar to those of *G. candidum* Gou1, *G. candidum* Que1, *G. candidum* Mah2, *G. candidum* CBS178.71, and *G. candidum* CBS11176. Strains MS1 and MS2 were not closely related to *G. reessii*, *G. citri-aurantii*, or *G. pseudocandidum.* The sequence analyses of strains MS1 and MS2 based on the ITS region indicated that these two strains were phylogenetically identified as *G. candidum* (Fig. 6).

Fig. 6

Discussion

Sixteen fungus-like colonies were isolated from Maizuru Bay surface sediment. Ten isolates belong to Ascomycota based on a phylogenetic analysis. Representatives of the genera

193 *Penicillium* and *Hypocrea* were the most diverse and abundant fungi isolated from marine
194 sponges [14]. In the present study, 5 strains in the genus *Hypocrea* were isolated from coastal
195 sediment. *Hypocrea* may be one of the most frequent genus isolated from marine
196 environments. Isolated marine fungi have previously been classified as belonging to the phyla
197 Chytridiomycota, Oomycota, Ascomycota, Basidomycota, and Zygomycota [13,14]. The
198 presently isolated fungi from the coastal marine sediment do not show substantial
199 phylogenetic divergence.

200 Phylogenetic 18S rRNA gene sequence analysis showed that strains MS1 and MS2 were
201 related with the genus *Geotrichum*, phylum Ascomycota, class Saccharomycetes, order
202 Saccharomycetales, family Endomycetaceae. The 26S rRNA D1/D2 and ITS sequence
203 analyses indicated that strains MS1 and MS2 were phylogenetically identified as *G. candidum*.
204 *G. candidum* is known as a plant pathogenic fungus and is used for rind formation during
205 Camembert cheese production [21, 22]. *Geotrichum candidum* refers to an anamorph and
206 *Galactomyces candidus* refers to a teleomorph [19, 23]. In the present study, strain MS1 is
207 designated *Geotrichum candidum* MS1, and strain MS2 is designated *Geotrichum candidum*
208 MS2. There could be confusion regarding whether *Galactomyces candidus* and *Galactomyces*
209 *candidum* is the appropriate species name. The original species names used in the associated
210 references or gene databases are used in this paper.

211 Liquid cultures and agar plates of strains MS1 and MS2, respectively, produced a

melon-like aroma. Volatile analysis indicated that the melon aroma resulted from acetones, non-acetone esters, sulfur-containing compounds, alcohols, and aldehydes [24]. Important chemicals known to cause a melon aroma are cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal [24, 25]. GC analysis suggested that strains MS1 and MS2 produce these four major melon-aroma chemicals. *G. candidum* isolated from sludge of an aerated pilot-scale bubble column was reported to produce a pineapple-like aroma [26]. It produces ethyl esters of acetic acid and butyric acid, methyl-3-butan-1-ol, and methyl-2-propan-1-ol with glucose [26]. Strains MS1 and MS2 produce cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal; therefore, different strains of *G. candidum* could produce different kinds of compounds associated with fruity aromas. Production of volatile compounds by *Geotrichum fragrans* using cassava wastewater as substrate has also been reported [27]. The newly isolated fungal strains of MS1 and MS2 might be applicable for melon-aromatic food.

G. candidum directly and positively contributes to cheese ripening and flavor development of many soft and semi-hard cheeses [28, 29]. In Camembert cheese production, *G. candidum* grows on the outside of the cheese and contributes to the formation of a rind [22, 30]. ITS region sequence analysis suggested that strain MS1 is similar to *G. candidum* Tom1. Strain Tom1 was isolated from hard cheese, Tomette des Alpes, in France [31]. ITS region sequence analysis suggested that strain MS2 is similar to *G. candidum* Thu1. Strain Thu1 was isolated

from soft cheese, Thurgauer weinkäse, in Switzerland [31]. ITS sequences of *G. candidum* strains L13PC, L19PB, L20BK, and 282A were reported from cheese-related studies (unpublished data recorded in DDBJ/EMBL/GenBank). Strains MS1 and MS2 are closely related to *G. candidum* strains isolated from cheese; therefore, there is a possibility of using the presently isolated two yeast strains for cheese ripening. *Galactomyces* Ferment Filtrate is used to produce cosmetics such as facial treatment essence, clear lotion, and masks. It is reported to have markedly increased caspase-14 expression [32]. Capase-14 expression prevents epidermal UVB damage and water loss [33]. Phylogenetic information regarding this *Galactomyces* sp. is not available; therefore, further research is necessary to determine whether the ferment filtrates of strains MS1 and MS2 have similar activity.

The present study confirmed that marine environments harbor a diversity of unknown fungi with unique features, and further physiological researches on marine fungi are very important for applied and environmental microbiology. Considering industrial application in Japan, it is advantageous that the fungal strains of MS1 and MS2 with melon-aroma were newly isolated from Japanese territorial sea.

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343 protects against epidermal UVB photodamage and water loss. Nat Cell Biol 9:666–674
344

345

346 Table 1 List of primers used in this study

Primer name	Sequence (5'-3')	Amplification target	Reference
NS1	GTAGTCATATGCTTGTCTC	18S rRNA gene	17. Chen *et al.* 2011
Fungi18S-R	GATCCCTAGTCGGCATAGTT	18S rRNA gene	17. Chen *et al.* 2011
NL1	GCATATCAATAAGCGGAGGAAAAG	26S rRNA gene D1/D2 region	18. Yalçın *et al.* 2014
NL4	GGTCCGTGTTTCAAGACGG	26S rRNA gene D1/D2 region	18. Yalçın *et al.* 2014
ITS1	TCCGTAGGTGAACCTGCGG	IST region	19. Hong et al. 2004
ITS4	TCCTCCGCTTATTGATATGC	IST region	19. Hong et al. 2004

347

Figure legends

Fig. 1 Photographs of fungus-like colonies of strains MS1-MS16 isolated from the Maizuru Bay marine sediment.

Fig. 2 Phylogenetic tree including 10 isolated fungal strains from the Maizuru Bay marine sediment and authentic fungal strains based on the 18S rRNA gene sequences. The tree was constructed using the neighbor-joining algorithm with bootstrap analyses (1,000 replicates) using MEGA6. Accession numbers were shown in parentheses. The scale bar represents 0.05 of estimated sequence divergence. *S. imitatum* was used as an outgroup.

Fig. 3 Photographs of the cells of strain MS1 (a) and strain MS2 (b). These were photographed at magnification of 1,000 times by an optical microscope.

Fig. 4 Gas chromatograms of culture head gases for strains MS1 (a) and MS2 (b). Four chemicals, cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal, known as the main components of melon aroma were used for external standards.

Fig. 5 Phylogenetic tree for isolated strains MS1 and MS2 generated from 26S rRNA gene D1/D2 region sequences. The tree was constructed using the neighbor-joining algorithm with bootstrap analyses (1,000 replicates) using MEGA6. *Gal*. means genus *Galactomyces*, and *Geo*. means genus *Geotrichum*. Accession numbers were shown in parentheses. The scale bar represents 0.01 of estimated sequence divergence. *D. tetrasporeus* was used as an outgroup.

Fig. 6 Phylogenetic tree for isolated strains MS1 and MS2 generated from ITS region

sequences. The tree was constructed using the neighbor-joining algorithm with bootstrap analyses (1,000 replicates) using MEGA6. *Gal*. means genus *Galactomyces*, and *Geo*. means genus *Geotrichum*. Accession numbers were shown in parentheses. The scale bar represents 0.02 of estimated sequence divergence. *D. tetrasporeus* was used as an outgroup.

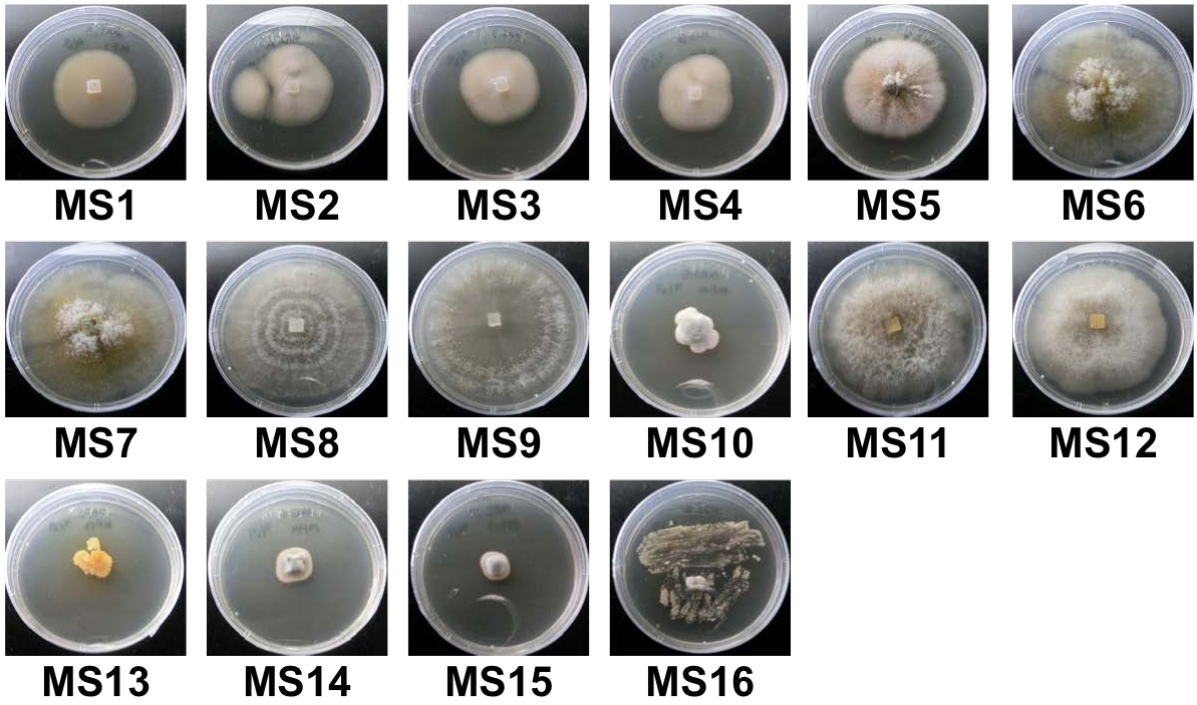

Fig. 1 Sutani et al.

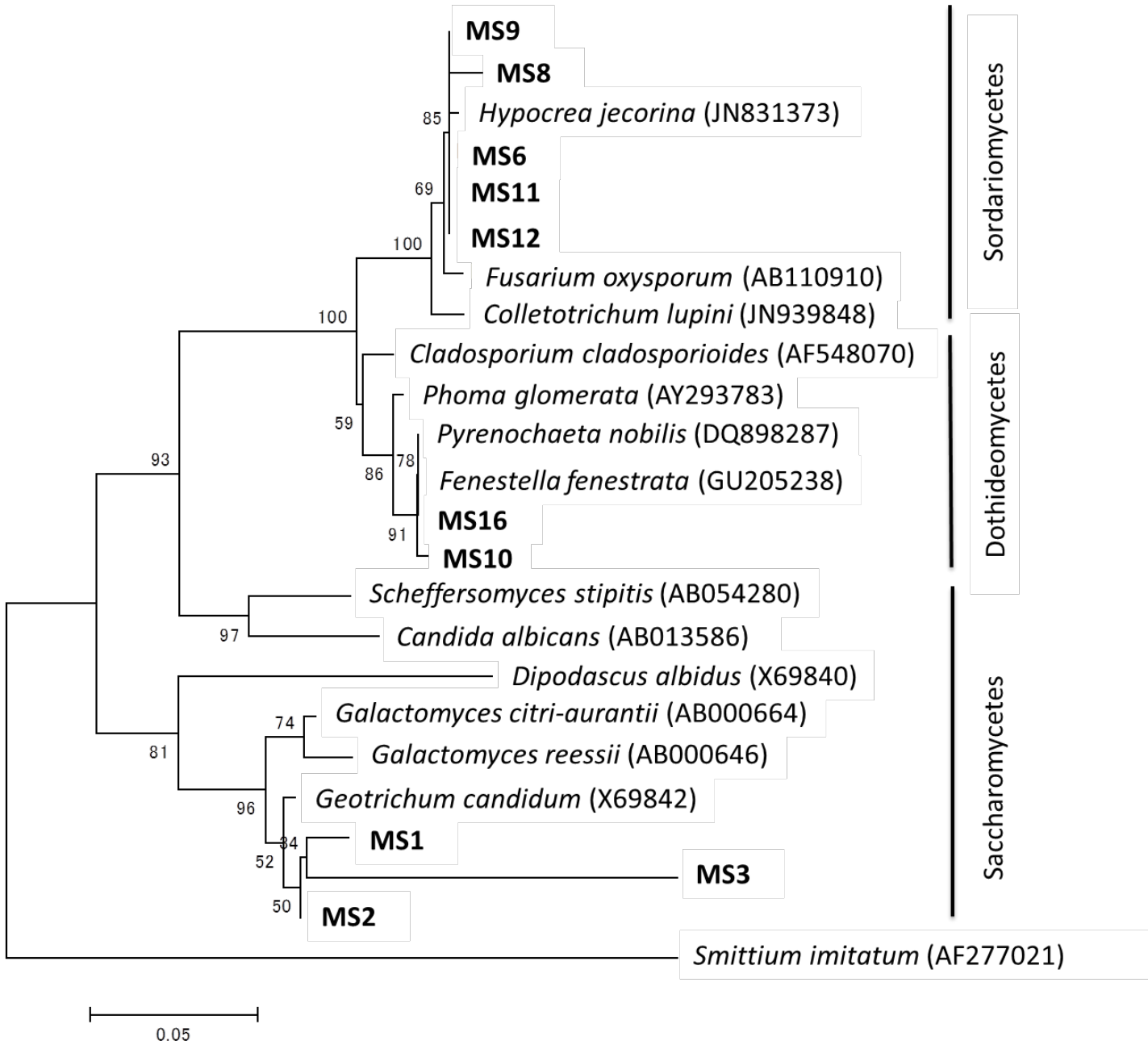

Fig. 2 Sutani et al.

a

b

Fig. 3 Sutani et al.

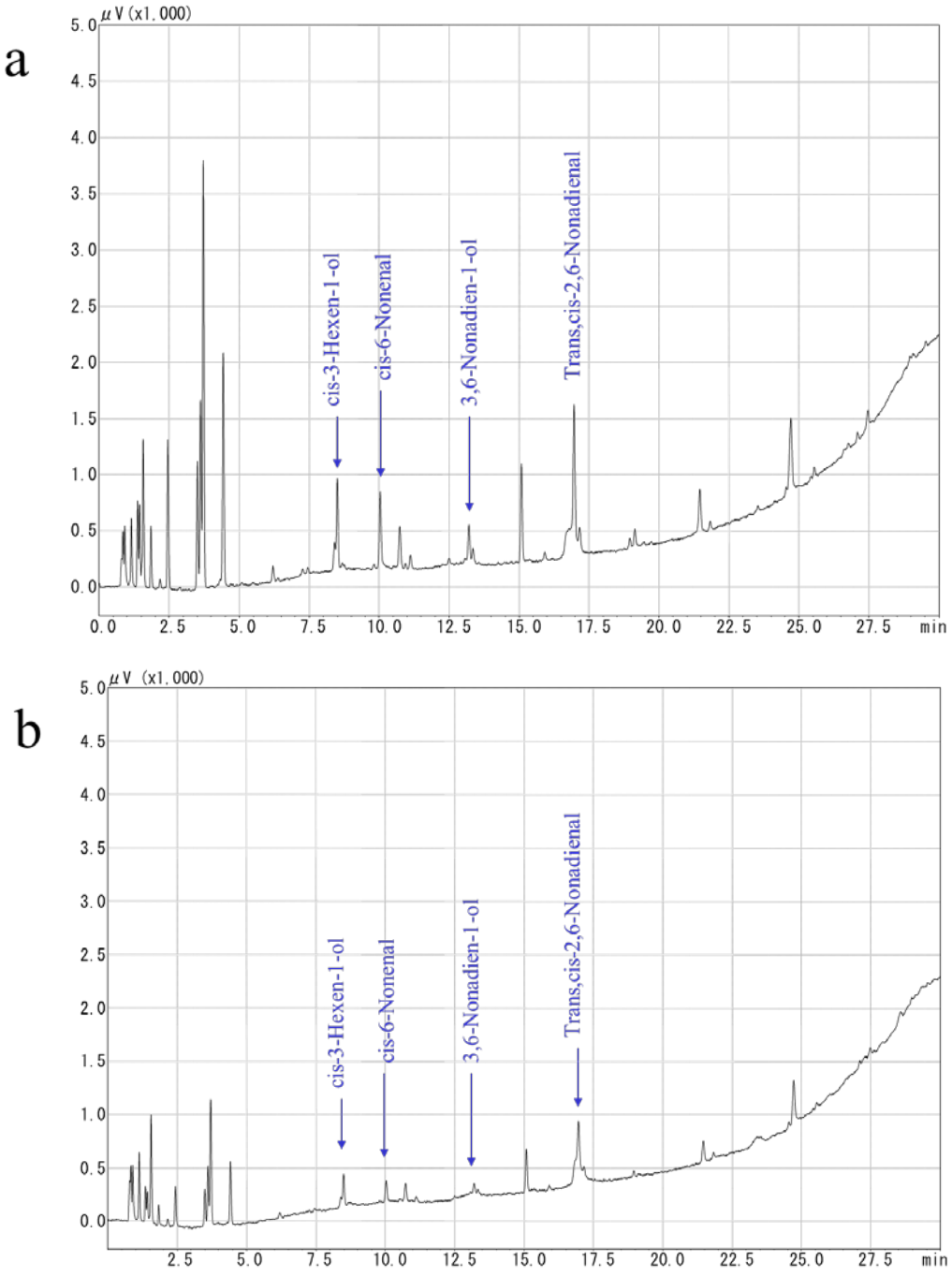

Fig. 4 Sutani et al.

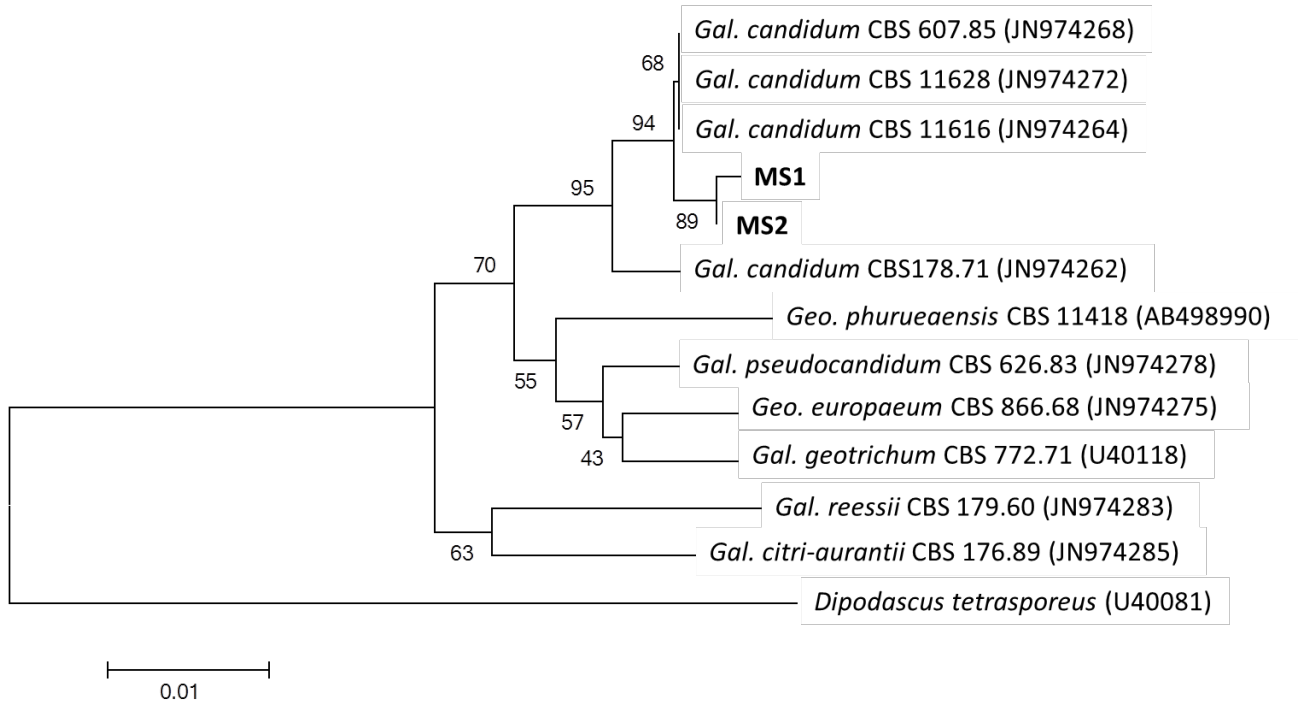

Fig. 5 Sutani et al.

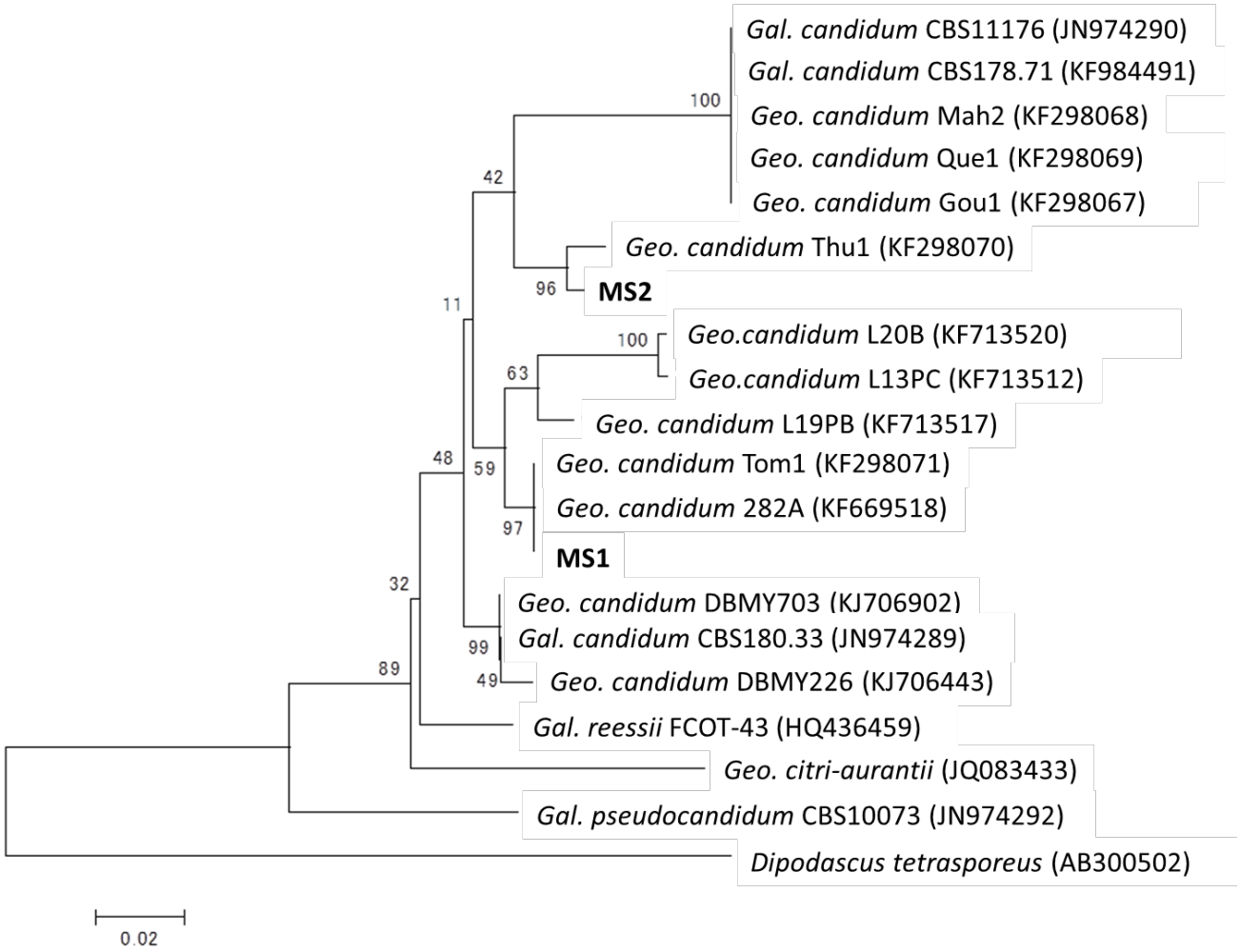

Fig. 6 Sutani et al.